

DETECTION OF KI-67, BCL-2, TERMINALLY APOPTOTIC CELLS (TUNEL ASSAY), CD34 AND VEGF IN AMELOBLASTOMA

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Abstract

Background: Ameloblastoma is a benign local aggressiveness odontogenic tumor that has great tendency to recur. **Aims:** characterize the staining profiles of various markers in ameloblastoma.

Materials and methods: immunohistochemical analysis of Ki-67, Bcl2, CD34 and VEGF and TUNEL assay were performed in 50 ameloblastomas. Mann-Whitney and Pearson's correlation applied for analysis. $P < 0.05$ was considered statistically significant.

Results: The outer odontogenic epithelial cells of ameloblastoma had low Ki-67 labeling index (3.66 ± 4.39) that is significant differed among its subtypes, and high Bcl2 expression in all variants (74.39%) and lining cells of unicystic ameloblastoma. On the other hand, inner odontogenic epithelial cells of ameloblastoma had low apoptotic index mean (3.5%), nevertheless high values were observed in acanthomatous (7.76%) and follicular (6.1%) subtypes. The mean count of new micro-vessels was (22.4 ± 25.54). They were predominantly round-shaped small-sized vessels. VEGF was highly expressed in the outer cells, and correlated with microvessels' count ($r=0.35$). VEGF expression did not relate to histological variation.

Conclusions: Ameloblastomas have high-strong expression for both Bcl2 and VEGF in outer tumor cells, and aberrant stromal neo-vascularization, but Ki67 expression significantly differed among their variants. Unicystic ameloblastoma should be considered with caution, their lining cells showed features of aggressiveness. It is recommended to evaluate Bcl2 and MvD to predict aggressiveness and VEGF to evaluate potential tumor survival and growth that may poses a possible benefit in treatment planes.

Keywords: Ameloblastoma, apoptosis, Bcl2, CD34, MvD, VEGF

Introduction

Ameloblastoma (AB) is a benign odontogenic tumor, with prevalence equal to or exceeding the combined total of all other odontogenic tumors excluding odontoma. It is usually seen in adults (relatively uncommon in teenagers) at posterior mandible [Waldron, 2009 and Rastogi et al, 2010]. AB is slowly growing, locally aggressive tumor that has great tendency to recur if not adequately excised and it has an unpredictable tendency to metastasize [Waldron, 2009; Rastogi et al, 2010; Amzerin et al, 2011; Ponniah, 2011].

According to the WHO classification, AB had several variants; solid/multicystic (SAB), extraosseous ameloblastoma, desmoplastic and unicystic type (UAB), beside malignant ameloblastoma and ameloblastic carcinoma [WHO, 2005]. Histologically AB is highly polymorphic, it has the ability to undergo various forms of metaplasia (acanthomatous, granulomatous, desmoplastic, basaloid, keratoameloblastoma and clear-cell ameloblastoma) [Waldron, 2009].

Molecularly AB gains growth and invasion potential through overexpression of TNF- α , anti-apoptotic proteins (Bcl2-Bcl-xL), and interface proteins (TGF, matrix metalloproteinase). ABs, however, have a low proliferation rate and P53 gene mutation do not appear to play a role [Regezi et al, 2008]. Researchers indicated that AB has higher proliferative activity than other benign odontogenic tumors [Sandra et al, 2001; El Deeb, 2003; Payeras et al, 2007; Yu 2007; Gadbail et al, 2011; Salehinejad et al, 2011; Razavi et al, 2012 and Florescu et al, 2012], that unrelated to its clinical behavior [Abdel-Azimet al, 2001 and, Salehinejad et al, 2011], although it can be used to differentiate ameloblastic carcinoma from AB [Kamath et al, 2010] or primary from recurrent AB [Piattelli et al, 1988; Migaldi et al, 1998 and Hirayama et al, 2004]. On the other hand, the reported high Bcl2 expression in some studies [Jie Wang et al, 2006 and Razavi et al, 2009] was contradicted by Mashhadiabbas and Najirad [2007] who also stated that Bcl2 can't be used to differentiate among benign, malignant or recurrent AB. Furthermore, other researchers did not find differences in VEGF expression between SAB and UAB [Handeet al, 2011]. Lastly, microvessel density (MvD) were reported to increase gradually in primary [Seifiet al, 2011], recurrent and malignant AB [Kumamoto et al, 2002; Zhong and Wang, 2003].

This study aimed to characterize the IHC staining profile of different types of AB by examining the expression and distribution of Ki-67, Bcl-2, CD34 and VEGF beside localization and detection of terminally apoptotic

cells, hopping to understand the biological features behind their polymorphic metaplasia.

Materials and methods

A total of 50 formalin fixed paraffin embedded blocks of AB and their files were collected from the archives in the Department of Oral Pathology/University of Baghdad/College of Dentistry. Serial 4µm sections were cut for routine and IHC staining. Cases were re-examined and diagnosed according to the WHO [2005]. The study was approved by the ethics committee of the college.

Sections were deparaffinized and rehydrated, for IHC they were immersed in antigen retrieval solutions (BioGenex: Citra for Ki-67 and Bcl2, Chemicon: EDTA pH 8.0 for VEGF) and subjected to autoclave pretreatment at 121 °C (20, 3 and 5 min. for Ki-67, Bcl2 and VEGF respectively). For CD34 this step was not recommended. Endogenous peroxidase activity was blocked by 0.3% H₂O₂ (30 min). Then sections were incubated with 4 primary monoclonal antibodies; mouse anti Ki-67 (BGX-297, BioGenex, USA; ready to use; 2hrs at 37°C), mouse anti-Bcl2 (BioGenex, USA; ready to use; 1hr at 37°C), anti-VEGF (Chemicon, Germany, diluted at 1:50, 1hr at 37°C) and mouse anti-CD34 (ImmunoTech, France, diluted at 1:50, 1hr at 37°C). Section treated with secondary antibody for 30 min at 37°C, then allowed to react with streptavidin-horseradish peroxidase reagent (BioGenex, B-SA system) or streptavidin AP reagent (Chemicon immunophosphatase detection system) for 30 min at 37°C, and reaction products were visualized either by diaminobenzidine (DAB) or fast red chromogen for 20 min then counter stained with hematoxylin. Negative control sections were treated with phosphate buffer saline (PBS) instead of the primary antibody.

From each section 5 representative fields were selected and a minimum of 1000 positive neoplastic odontogenic epithelial cells were evaluated at a magnification of X400. All slides were assessed blindly by two pathologists and the average of readings was obtained. The mean Ki-67 labeling index (LI) was calculated and the remaining antibodies were evaluated semi quantitatively as follows: Bcl2 expression: negative ≤5%; +: > 5-20%, ++: > 20-50% and +++: > 50-100% [Chrysomali et al, 2003]. VEGF scored as; +: 0-19%, ++: 20-39%, ++: 40-59% and +++: 60-100% and its intensity assessed as: weak, moderate and strong [Lim et al, 2003]. The MvD is equal to the number of CD34 positive vessels in 5 hotspot fields of stroma at high power (X400) [Vermeulen et al, 1996]. Additionally the shape of microvessels was classified into round and elongated, whereas the size was assessed subjectively as small (contain ≤ 3 RBCs or endothelial cell

clusters or single cell sprout not luminated) and medium vessels (contain > 4 RBCs or dilated lumen).

For detection of apoptotic DNA fragmentation ApopTag plus peroxidase in situ apoptotic detection kit code S7101 (Chemicon, Germany) was used. New sections were deparaffinized, then treated with 20µg/ml proteinase K (15 min) and with 3% H₂O₂ in PBS to eliminate endogenous peroxidase activity. Subsequently they were incubated with TdT (1hr, 37°C) followed by anti-digoxigenin conjugate (30 min). Then immersed in a 0.03% DAB (3 min) and counterstained with methyl green, finally dehydrated and mounted.

Apoptosis index (AI) from 5 randomly selected representative fields at high power (X400) was performed. The stage of apoptosis depending on nuclear morphology of positive cells was identifying as: early staged: round nuclei with chromatin margination; middle stage: condensed nuclei; and late stage: nuclear fragmentation (apoptotic bodies) at (X1000).

SPSS statistical software was used to perform Mann-Whitney test and Pearson's correlation coefficient. Probabilities of less than 0.05 were accepted as significant.

Results

AB occurred in patients aged between 5-85 years, predominantly in males, at posterior region of the mandible (94%). 24 cases were SAB and 26 cases were UAB (16 cases of them had mural growth).

Nuclear Ki-67 positivity was detected in 88% of the cases, predominately at outer cells with 3.66 ± 4.39 LI (table-1). Ki-67 expression was less in females and it correlated with age ($r=0.3$, $p=0.018$) (table2). Ki67 expression significantly differed among AB variants (table 3). The highest value was reported in basal type (15.45) (figure-1A). Follicular islands whether they were in SAB or in mural growth of UAB (figure-1B) showed similar Ki-67 expression.

Ameloblastomas showed high cytoplasmic Bcl2 expression (74.39%) with strong intensity at the outer cells in all cases, particularly in follicular SAB (75.8, figure-1C), mural islands of UAB (84.1, figure-1 D) and lining cells of UAB (73.0). Almost all inner cells had low (23.62) and weak Bcl2 expression (table1), except those in acanthomatous variant (table-3). Male patients had significantly higher Bcl2 expression at both the inner (22.5, $P=0.02$) and lining cells (76.73, $P=0.03$). Patients with age ≤ 20 had lowest Bcl2 positivity (10.8 ± 15.7) in inner cells. Bcl2 expression significantly correlated with Ki-67 at outer cells ($r= -0.45$) and in UAB it had stronger correlation at both outer and lining cells ($r=-0.63$, $r=-0.66$ respectively). Thus UAB differ from other variants by strongly maintaining cells surviving in different compartment; mural and lining.

TUNEL reaction was positive in 44 (88%) cases predominantly in inner cells (3.5 ± 3.39 , figure-1E) and to lesser extent in lining cells of UAB (1.35 ± 1.17) (table 1). The later showed high AI in females (2.1 ± 1.3) and in patients older than 40 years (2.06 ± 1.72 , table 2). Histologically, acanthomatous (figure-1F) and plexiform AB expressed significantly high AI, especially when compared with mural islands of UAB (7.76, $P=0.026$ and 6.1, $P=0.045$ respectively versus 1.58). Desmoplastic variant did not express TUNEL staining (table-3). Concerning stage of cell death, acanthomatous AB had high number of cells at late fragmentation stage (46.33%), while plexiform showed more cells at early stage of apoptosis (margination, 43.33%, $p=0.01$). The remaining variants were in mixed stages of cell death (table 3). AI correlated with Bcl2 at inner cells, outer cells and lining cells ($r=-0.814$, $r=-0.43$ and $r=-0.99$ respectively).

VEGF showed significant high positive cytoplasmic and membranous expression with strong intensity in outer and lining tumor cells as well as in endothelial cells in all cases. Patients with >40 years old showed significantly less expression with weak intensity in lining cells of UAB (69.13, $P=0.031$) and in inner cells (9.16, $P=0.05$) respectively (Table 2).

VEGF positivity in the outer cells did not differ among AB variants (figure-2A), and the highest positivity was recorded in mural islands of UAB (93.7, table 3), while its intensity was almost strong except in plexiform and lining cells of UAB they were of moderate intensity (66.7%, and 52%, respectively, $P<0.05$). Regarding the inner cells, squamous metaplastic cells had significantly highest positivity (55) with 100% strong intensity.

VEGF expression correlated with Ki-67 expression at both the outer ($r=0.34$, $p=0.05$) and inner cells ($r=-0.991$, $p=0.00$) and with Bcl2 at inner cells ($r=0.423$, $p=0.05$).

The mean count of new microvessels (MvD) in AB was (22.4 ± 25.54). They were predominantly round-shaped vessels and small-sized (table 4, figure-2B) with no statistical sex differences. Patients below 20 years old expressed significantly the highest MvD around tumor islands than other age groups (35.97 ± 47.7 , $P=0.03$, table 2).

The mean MvD did not differ among SAB variants (table 3), but the single case of desmoplastic AB showed very high number of small vessels. Whereas plexiform AB showed significantly fewer small vessels (3 ± 1.99 , $P=0.02$), and had elongated medium size vessels (table 3).

MvD around tumors' islands correlated significantly at outer cells with the expression of Ki-67 ($r=0.45$, $p=0.014$) and Bcl2 ($r=0.5$, $p=0.042$) and it correlated significantly with AI at inner cells ($r=-0.319$, $p=0.05$). The microvessels' size correlated with Bcl2 expression at outer cells of SAB islands ($r=0.56$, $p=0.03$) and lining cells of UAB ($r=0.567$, $p=0.04$). The number of round shape new vessels correlated with VEGF expression in

outer cells ($r=0.32$, $p=0.035$) of ameloblastic islands and lining cells of UAB ($r=0.357$, $p=0.037$).

Discussion

Ameloblastomas deserves special attention, not only because of its particular biological behavior but also due to its relatively high frequency among odontogenic tumors. Estimation of IHC staining profile in different AB variants could be helpful in understanding the biological features behind their polymorphic metaplasia, and may poses a possible benefit in treatment planes or establishment of prognostic value in the future.

Similarly to previous studies [Sandra et al, 2001; Razavi et al 2012; Florescu et al, 2012; Amaral et al, 2012] we indicate that the majority of AB had low Ki-67 LI (3.66) and it was lesser than that reported by Abdel Aziz (8.29 ± 3.15) [Abdel-Aziz and Amin 2012] who remarked to even a higher LI in recurrent cases (LI=19). Furthermore females and younger age patients expressed lower proliferative activity. These results support Sandra et al [2001] findings that lowest values being present in young patients and vice versa.

We agree with previous reports that the cellular proliferation and consequently AB growth are related to outer cells [Sandra et al, 2001; Bologna-Molina et al, 2008; Florescu et al, 2012, Amaral et al, 2012; Abdel-Aziz and Amin, 2012] and that follicular AB has higher Ki-67 expression than plexiform [Sandra et al, 2001; Kumamoto et al, 2002; Yu, 2007; Florescu et al, 2012], but we contradict, Florescu et al [2012] by observing Ki-67 expression in granular AB. Furthermore, this study showed a high Ki-67 expression in both basal and acanthomatous AB, and almost scant proliferation in desmoplastic AB (although cases number was limited). Thus proliferation assessment may be related to epithelial island size rather than related to its aggressiveness. On the other hand, UAB had lower proliferative activity than SAB, but it become identical or slightly higher than follicular AB when it had mural growth. This explains why Bologna-Molina et al [2008] found both types had nearly similar proliferative activity. Yet other studies did not correlate Ki-67 expression with AB variants [Mashhadiabbas et al, 2007, Migaldi et al, 2008; Bello et al, 2009] but rather related it to relapses [Migaldi et al, 2008].

Accordingly AB showed limited proliferative activity if compared to its clinical locally invasive behavior, support the concept that infiltrating potential of AB is not related to the cellular proliferation index [Abdel-Azim et al, 2001, Bello et al, 2009; Salehinejad et al, 2011]. This will not denote such index may be higher if samples are taken selectively from invasive front or designing long cohort studies to confirm its causal relationship with clinical behavior.

Bcl2 expression was observed in 100% of cases, predominantly at outer cells with greater positivity and strong intensity than previously reported (50% and 88.2%) [Luo et al, 2006; Florescu et al, 2012]. While, AI for terminal apoptotic cells was observed in 88% in the inner cells. In contrast to our results, Amaral et al [2012] demonstrated more apoptotic cells in outer cells than in inner areas of AB and at much higher level 3.5 versus 18.8. We found an inverse correlation between AI and Bcl2 expression, but both of them had no relation with sex or age variation.

Although it is believed that UAB has an innocent behavior, in this study however, its outer and lining cells seem to resist apoptosis by harboring great Bcl2 expression than plexiform, basaloid and the acanthomatous AB. Furthermore, outer cells of mural islands process even higher and strong Bcl2 expression than follicular and plexiform. Inner cells of SAB generally express moderate and weak Bcl2, but at early squamous metaplasia (without keratin) they showed high expression, that changed to be scant and associated with high AI when they become well keratinized (indicating their death). On the other hand, granular transformed cells showed many apoptotic cell fragments with condensed nuclei suggesting great late stage of cell death that associated with phagocytosis by neighboring neoplastic cells. This observation coincides with Kumamoto et al [2001] but contradicts Florescu et al [2012] who did not report Bcl2 expression in granular or squamoid cell. Lastly, Mashhadiaddas et al [2007] could not use Bcl2 to differentiate between different AB variants and Luo et al [2006] did not attribute Bcl2 over expression to AB aggressiveness or recurrent behavior.

Few studies were available concerning angiogenesis in AB. In this study, AB showed strong and high VEGF expression in outer cells of all AB variants with weak positivity in inner cells. Males and old patients had lower VEGF expression at inner and lining cells of SAB and UAB respectively. On the other hand, endothelial cells of the microvessels showed weak VEGF expression. This indicates that odontogenic epithelia induce angiogenesis, via paracrine mechanism [Kumamoto et al, 2002] and play a critical role in tumor growth and it might also work as an autocrine growth factor, if it is proved to have VEGFR. Inner cells with squamous metaplasia expressed the highest VEGF with strongest intensity while inner cells in basaloid, desmoplastic, granular and UAB showed least positivity thus contradicting Kumamoto et al [2002] results who stated that keratinizing and granular cells in AB showed low VEGF expression. Furthermore, mural islands in UAB showed over expression of VEGF in the outer cells.

The current study also illustrated that MvD in tumor stroma was higher than that reported around tooth germ (unpublished data) and related to VEGF expression, just in line with Kumamoto et al [2002] and Zhong study

[2003]. It also confirms that increase MvD is important in AB progression and invasiveness [Gadbail et al, 2012] in all histological variant irrespective to sex and age. Therefore measurement and assessment of angiogenesis in AB can be valuable in predicting response to antiangiogenic therapeutic strategies and also provide objective assessment of post therapeutic response. Such therapy may aid in the regression of the tumor mass and preventing expansion, so decreasing the chance for aggressive radical treatment and reducing the risk of disability.

Furthermore, aberrant neo-vascularization is reported in this study in AB stroma, thus confirm Gadbail et al [2012] observation for the evidence of slightly dilated and tortuous microvessels. Similar to Seifi et al [2011], but in contrast to Kumamoto et al [2002] findings, there were noticeable differences in both MvD and blood vessel size between follicular and plexiform AB. Microvessels were numerous, rounded and small in follicular AB, whereas, they were elongated of medium size in plexiform. Basal cell AB showed diffused expression of VEGF in most tumor cells, despite the MvD was fewer than other subtypes; the latter finding was similar to Seifi [2011]. Therefore, the expression of CD34 and assessment of MvD may be determining factors in prognosis and prediction of AB progress.

This study indicated that VEGF is highly correlated with Ki-67 expression at outer cells, and with Bcl2 at inner cells.

In conclusion most variant of AB characterized by low Ki-67 expression (limited growth) and high-strong expression for Bcl2 and VEGF (great tumor cells' survival) at outer cells of tumor islands. According to correlations' results, increase VEGF leads to inhibition of apoptosis, through the induction of expression of anti apoptotic protein bcl2 (even in inner cells) with increase in the proliferative activity of the outer cells that secrete this growth factor, and induct the formation of more blood vessels in the stroma. More blood vessels, may further exaggerate the picture, if they are well established as numerous luminated vessels and then they further will increase the proliferation in the outer cells as well as increase survival of the outer and inner cells and reduce death of cells. In spite of that, outer cells neither die nor proliferate and maintain tumor mass survival. Therefore, it is recommended to evaluate Bcl2 positivity and intensity in tumor cells with MvD around tumor islands instead of looking for proliferation. Further comparison with other odontogenic neoplasm beside clinical data (recurrence or high grade transformation) the above parameters may give more practical value.

References:

Abdel-Azim AA, Mostafa KA, Ismail MT, Abo-Donia MA. Expression of argyrophilic nuclear organizer regions (AgNORs) and ki-67 in

ameloblastomas and adenomtoid odontogenic tumors (AOTs). Egypt Dent Asso 2001; 47: 1929.

Abdel-Aziz A, Amin MM. EGFR, CD10 and proliferation marker Ki67 expression in ameloblastoma: possible role in local recurrence. Diagnostic Pathol 2012; 7: 14.

Amaral FR, Mateus GCP, Bonisson LA, De Andrade BAB, Mesquita RA, Horta MCR, De Andrade H. Cell proliferation and apoptosis in ameloblastomas and keratocystic odontogenic tumors. Braz Dent J 2012; 23: 91-96.

Amzerin M, Fadoukhair Z, Belbaraka R, Iraqui M, Boutayeb S, M'rabti H, Kebdani T, Hassouni K, Benjaafar N, El Gueddari BK and Errihani H. Metastatic ameloblastoma responding to combination chemotherapy: case report and review of the literature. J Med Case Reports 2011;5: 491.

Bello IO, Alanen K, Slootweg PJ, Salo T, Alpha-smooth muscle actin within epithelial islands is predictive of ameloblastic carcinoma. Oral Oncol 2009; 45:760–765.

Bologna-Molina R, Mosqueda-Taylor A, Lopez-Corella E, et al. Syndecan-1 (CD138) and Ki-67 expression in different subtypes of ameloblastomas. Oral Oncol 2008; 44: 805–11.

Chrysomali E, Nikitakis N, Tosios K, Sauk J, Papannicolaou S. Immunohistochemical evaluation of cell proliferation antigen Ki-67 and apoptosis-related proteins bcl2 and caspase-3 in oral granular cell tumor. Oral Surg Oral Med Oral Pathol Oral Radiol Endod. 2003; 96:566-72.

El Deeb AM, Moustafa WA. Expression of cell proliferation markers (PCNA and AgNOR) in odontogenic cysts and ameloblastoma. Egyptian Dent J 2003; 49: 1563.

Florescu A, Simionescu C, Ciurea R, and Pitru A. P53, Bcl-2 and Ki67 immunoexpression in follicular solid ameloblastomas. Rom J Morphol Embryol 2012; 53: 105-109.

Gadbail AR, Gadbaile MPM, Hande A, Chaudhary MS, Gondivkar SM, Korde S, Gawande MN, Patil S, Tekade S, Sharma A. Tumor angiogenesis: Role in locally aggressive biological behavior of ameloblastoma and keratocystic odontogenic tumor. Head & Neck March 20, 2012; DOI: 10.1002/hed.22960: available at: <http://www.ncbi.nlm.nih.gov/pubmed/22431258> (accessed Jan 20, 2013).

Gadbail AR, Patil R, Chaudhary M. Co-expression of Ki-67 and p53 protein in ameloblastoma and keratocystic odontogenic tumor." Acta Odontologica Scandinavica 2011. (doi:10.3109/00016357.2011.600714)

Hande AH, Gadbaile AR, Sonone AM, Chaudhary MS, Wadhwan V, Nikam A. Comparative analysis of tumor angiogenesis in solid multicystic and unicystic ameloblastoma by using CD 105 (endoglin). Archives Oral Biol 2011; 56: 1635-1640.

- Hirayama T, Hamada T, Hasui K, Semba I, Murata F, Sugihara K. Immunohistochemical analysis of cell proliferation and suppression of ameloblastoma with special reference to plexiform and follicular ameloblastoma. *Acta Histochem Cytochem* 2004; 37: 391–8.
- Irulandy Ponniah. Recurrent unicystic ameloblastoma in a child. *J Oral Maxillofac Pathol*. 2011 May-Aug; 15(2): 236–238.
- Jaaskelainen K, Jee KJ, Leivo I, Saloniemi I, Knuutila S, Heikinheimo K. Cell proliferation and chromosomal changes in human ameloblastoma. *Cancer Genet Cytogenet* 2002; 136: 31–7.
- Jie Wang JM, Ming Z, Liu J. Expression and significance of Bcl-2 family in ameloblastoma. *Chin J Cancer Res* 2006;18:149-53.
- Kamath KP, Vidya M, Shetty N, Karkera BV, Jogi H. Nucleolar organizing regions and α -smooth muscle actin expression in a case of ameloblastic carcinoma. *Head & Neck Pathol* 2010; 4: 157–162.
- Kumamoto H, Ohki K, Ooya K. Association between vascular endothelial growth factor (VEGF) expression and tumor angiogenesis in ameloblastomas. *J Oral Pathol Med* 2002; 31: 28-34.
- Kumamoto H, Ooya. Immunohistochemical and ultrastructural investigation of apoptotic cell death in granular cell ameloblastoma. *J Oral Pathol Med* 2001; 30: 245-250.
- Lim J, Kang S, Lee M, Pai H, Yoon H, Lee J, Hong S, Lim C. Expression of vascular endothelial growth factor in salivary gland carcinomas and its relation to P53, Ki-67 and prognosis. *J Oral Pathol Med* 2003;32:552-61.
- Luo HY, Yu SF, Li TJ. Differential expression of apoptosis-related proteins in various cellular components of ameloblastomas. *Int J Oral Maxillofac Surg* 2006; 35: 750–5.
- Mashhadiabbas F, Najirad Z. Detection of P53, PCNA, Bcl-2 and Ki-67 in ameloblastoma. Shaheed Beheshti Medical University, Tehran, Iran. 0751. Seq#98 Oral Biology and Pathology, March 22 2007. Ernest N. Morial Convention Center Exhibit Hall I2-J. Available at : http://iadr.confex.com/iadr/2007orleans/techprogram/session_17080.htm. (accessed Jan 20, 2013).
- Migaldi M, Criscuolo M, Zunarelli E, Lo-Bianco L, Martinella A, Barbolini G. P120 and AgNOR protein expression: a comparison with nucleolar proliferation markers in oral pathology. *Oral Surg Oral Med Oral Pathol Oral Radiol Endod*. 1998; 85:189-96.
- Migaldi M, Sartori G, Rossi G, Cittadini A, Sgambato A. Tumor cell proliferation and microsatellite alterations in human ameloblastoma. *Oral Oncol*, 2008; 44:50–60.
- Payeras MR, Sant'Ana Filho M, Lauxen IS, Barbachan JJD. Quantitative analysis of argyrophilic nucleolar organizer regions and epidermal growth factor receptor in ameloblastomas. *J Oral Pathol & Med* 2007; 36: 99-104.

- Piattelli A, Fioroni M, Santinelli A, Rubini C. Expression of proliferating cell nuclear antigen in ameloblastomas and odontogenic cysts. *Oral Oncol* 1998; 34: 408–12.
- Rastogi V, Pandilwar PK, Maitra S. Ameloblastoma: an evidence based study. *J Maxillofac Oral Surg*. 2010; 9: 173-177.
- Razavi M, Tabatabaie H. Immunohistochemical study of Bcl- 2 and Ki-67 expression and apoptosis with tunel method in OKC in comparison with SAB. *J Isf Dent Sch* 2009;5:139-47.
- Razavi SM, Tabatabaie SH, Hoseini AT, Hoseini ET, Khabazia A. A comparative immunohistochemical study of Ki-67 and Bcl-2 expression in solid ameloblastoma and adenomatoid odontogenic tumor. *Dent Res J* 2012; 9: 192-197.
- Regezi JA, Sciubba JJ, Jordon RCK, editors. *Oral pathology, clinical pathologic correlations*. 5th ed. Saunders, Elsevier 2008; p. 261-7.
- Salehinejad J, Mahmoodabadi RZ, Saghafi S, Jafarian AH, Ghazi N, Rajaei AR, Marouzi P. Immunohistochemical detection of p53 and PCNA in ameloblastoma and adenomatoid odontogenic tumor. *J Oral Sci* 2011; 53: 213-217.
- Sandra F, Mitsuyasu T, Nakamura N, Shiratsuchi Y, Ohishi M. Immunohistochemical evaluation of PCNA and Ki-67 in ameloblastoma. *Oral Oncol* 2001; 37: 193–8.
- Sandra F, Nakamura N, Mitsuyasu T, Shiratsuchi Y, Ohishi M. Two relatively distinct patterns of ameloblastoma: an anti-apoptotic proliferating site in the outer layer (periphery) and a pro-apoptotic differentiating site in the inner layer (centre). *Histopathol* 2001; 39: 93–8.
- Seifi S, Shafaei S, Ghadiri S. Microvessel density in follicular cysts, keratocystic odontogenic tumors and ameloblastomas. *Asian Pacific J Cancer Prev* 2011; 12: 351-356.
- Vermeulen P, Gasparini G, Fox S. Quantification of angiogenesis in solid human tumors: an international consensus on the methodology and criteria of evaluation. *Eur J Cancer* 1996;32:2474-84.
- Waldron CA. odontogenic cyst and tumors. In: Neville BW, Dam DD, Allen CM, Bouquot JE, editors. *Oral and maxillofacial pathology*. 3rd ed. Saunders, Elsevier, 2009; p.702-11.
- WHO Classification of Tumours Series. In: Barnes L, Everson JW, Reichart P, Sindransky D, eds. *Pathology and genetics of head and neck tumors*. Lyon: IARC Press 2005.
- Yu-fang. The Study of Proliferating Activity in Ameloblastoma. MSc thesis in Oral clinical medicine: Master's institutions: Hebei Medical University, 2007.
- Zhong M, Wang J. Expression of CD34 and VEGF in human ameloblastoma and odontogenic keratocyst. *Chinese J Dent Res* 2003; 6: 14 – 19.

Table 1: The mean immunohistochemical expression of positive markers in ameloblastoma at different localization

| | No | Ki-67 | P value | Bcl2 100% | P value | TUNEL 88% | P value | VEGF 100% | P value |
|--------|----|-----------|---------|------------|---------|-----------|---------|------------|---------|
| Outer | 50 | 3.66±4.3 | 0.0001 | 74.39±16.6 | 0.001 | 0.99±0.99 | 0.01 | 81.7±15.5 | 0.02 |
| Inner | 50 | 0.03±0.11 | | 23.62±2.8 | | 3.5±3.39 | | 31.89±15.1 | |
| Lining | 26 | 1.15±1.44 | | 73.04±16.5 | | 1.35±1.17 | | 74.88±14.7 | |

Table (2) Mean of immunohistochemical expression of positive markers in ameloblastoma in relation to sex and age

| | | Sex | | P value | Age | | | P value |
|-------|---------------|-------------|-------------|---------|-------------|-------------|-------------|---------|
| | | Male | Females | | <20 | 21-40 | >40 | |
| Ki67 | Outer | 4.11±5.22 | 2.78±1.85 | | 2.63±2.09 | 3.4±5.18 | 4.3±4.02 | |
| | Inner | 0.25±0.12 | 0.42±0.1 | | 0 | 0.03±0.09 | 0 | |
| | Lining | 1.05±1.39 | 1.3±1.58 | | 1±0.78 | 0.99±1.03 | 1.21±2.06 | |
| Bcl2 | Outer | 73.17±18.46 | 74.07±15.73 | | 72.17±18.65 | 73.21±19.33 | 73.7±14.94 | |
| | Inner | 22.56±18.28 | 12.92±13.39 | 0.02 | 10.8±14.75 | 22.19±17.51 | 25.6±16.81 | |
| | Lining | 76.73±17.9 | 68±13.49 | 0.03 | 73.43±18.56 | 73.34±16.65 | 70±15.17 | |
| VEGF | Outer | 83.95±13.4 | 79.53±18.25 | | 84.11±14.52 | 77.87±16.4 | 85.7±12.55 | |
| | Inner | 14.28±12.6 | 29.28±15.39 | | 27.5±12.52 | 21.81±15.85 | 9.16±11.14 | 0.05 |
| | Lining | 69.13±14 | 82.72±14 | | 74.14±15.83 | 72±14.23 | 69.13±12.51 | 0.031 |
| TUNEL | Outer | 1.5±1.55 | 0.7±0.6 | | 0.3 | 1.2±1.018 | 0 | |
| | Inner | 3.68±3.31 | 3.26±3.58 | | 4.33±5.32 | 2.96±1.81 | 3.78±3.88 | |
| | Lining | 0.6±0.57 | 2.1±1.31 | 0.05 | 0.66±0.61 | 1.3±1.1 | 2.06±1.72 | |
| CD34 | Around island | 25.36±31.06 | 16.86±6.7 | | 35.97±47.7 | 17.99±9.51 | 16.48±3.9 | 0.03 |
| | Around lining | 13.01±4.04 | 13.16±4.2 | | 11.6±2.65 | 13.03±3.89 | 13.63±4.61 | |

Table 3: The mean immunohistochemical expression of positive markers in different ameloblastoma's histological subtypes at different cell locations

| | | Follicular (11) | Plexiform (6) | Acantho (3) | Basal (2) | Desmo (1) | Granular (1) | Mural islands UAB (19) | P value |
|-------|-----------|--------------------|------------------|----------------|--------------|--------------|-----------------|---------------------------|------------|
| Ki 67 | Outer | 2.85±1.9 | 1.5±1.07 | 9.36±2.27 | 15.45±10.67 | 0.9 | 1.4 | 2.97±3.48 | 0.03 |
| | Inner | 0.08±0.19 | 0 | 0 | 0 | 0 | 1.3 | 0 | |
| Bcl 2 | Outer | 75.8±16.1 | 56±13.8 | 55±2.12 | 50±18 | 70 | 60 | 84.1±10.57 | 0.02 |
| | Inner | 17.3±11.9 | 13.8±14.5 | 50±18 | 27.5±03.5 | 30 | 40 | 26±15.6 | |
| VE GF | Outer | 89.36±18.12 | 87.5±16.4 | 76.33±3.21 | 75±21.12 | 85 | 65 | 93.76±12.05 | |
| | Inner | 39±15.11 | 35±14.15 | 55±13.22 | 20 | 25 | 23 | 26.25±13.14 | |
| TUNEL | Outer | 0.85±0.77 | 1.95±1.9 | 0 | 0 | 0 | 0 | 0.62±0.54 | |
| | Inner | 3.65±2.85 | 6.1±5.48 | 7.76±1.9 | 2.95 | 0 | 3.4 | 1.58±1.43 | 0.03 |
| | Stage | Early | 13.36±18.71 | 43.33±37.75 | 19±24.75 | 16±4.24 | 0 | 15.22±12.33 | |
| | | Middle | 7±15.54 | 4.33±6.74 | 12.33±21.36 | 0 | 0 | 3.4 | 4.41±3.34 |
| | | Late | 15.±21.79 | 19.66±18.55 | 46.33±7.57 | 13.5±19.09 | 0 | | 14.55±7.28 |
| CD34 | Count | 20.61±10.58 | 40.11±67.43 | 16.67±1.44 | 16.4±12.44 | 38.8 | 8.4 | 19.25±5.97 | |
| | Round | 19.36±9.78 | 12.2±16.4 | 15.86±2.83 | 14.8±10.18 | 38.8 | 8.4 | 18.82±4.76 | |
| | Elongated | 11 ± 0.56 | 15±14 | 2.4±0 | | | | 8.43±6.83 | |
| | Small | 15.83±10.17 | 3±1.99 | 16.00±2.6 | 15.2±10.74 | 25.8 | 8.4 | 17.6±5.77 | 0.02 |
| | medium | 7.22±2.41 | 17.5±22.4 | 2±0 | 2.4±0 | 13 | | 7.42±5.22 | |

Table (4) Mean count, shape and size of CD34 positive microvessels around solid, mural and lining odontogenic epithelial tumor cell in ameloblastoma.

| Histological | No | Count | Shape | | Size | |
|--------------|----|------------|------------|-------------|------------|------------|
| | | | Round | Elongated | Small | Medium |
| Total | 50 | 22.4±25.54 | 18.42± 8.9 | 10.29±12.04 | 15.33±8.21 | 9.68±11.84 |
| Lining | 26 | 13.07±4.04 | 12.42±4.33 | 4.33±4.74 | 11.67± 4.7 | 4.17±1.65 |
| Mural | 19 | 19.25±5.97 | 18.82±4.76 | 8.43±6.83 | 17.6±5.77 | 7.42±5.22 |

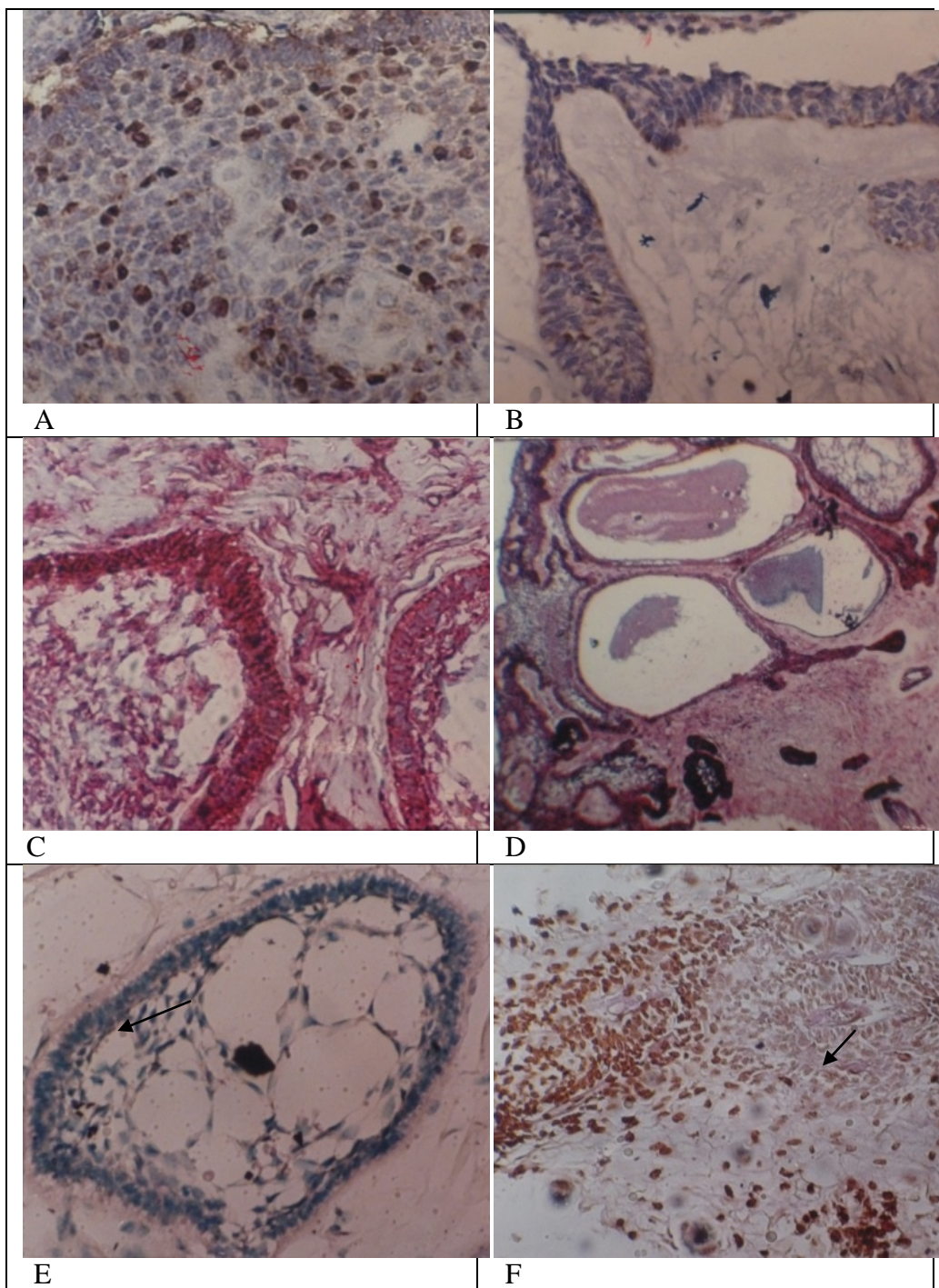
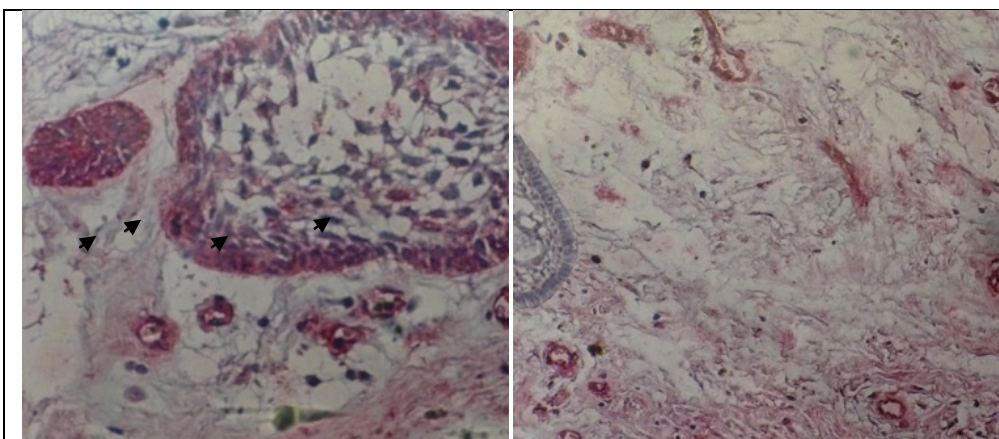


Figure 1: Immunohistochemical reactivity in odontogenic epithelium cells. Ki-67expression in basal ameloblastoma (A) and unicystic ameloblastoma (B)(X200). Bcl2 reactivity in outer cells and inner cells of follicular ameloblastoma (C, X200) and in lining and mural islands of unicystic ameloblastoma (D, X40). Positive TUNEL reaction in apoptotic cells (arrows) in the inner cells of follicular (E) and acanthomatous ameloblastoma (F) (X200).



A

B

Figure 2. Immunohistochemical reactivity for vascular endothelial growth factor in the outer cells of follicular ameloblastoma (A) and in the neighboring vessels of tumor stroma (arrowheads). (B) Immunohistochemical positive reactivity for CD34 in microvessels of surrounding stroma (X400).